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ANIMAL AND PLANT HEALTH INSPECTION SERVICE
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SAM 126

9 CFR 113.XXX	September 1, 1995	Bovine Rotavirus
Standard Requirement	New	Agent

SUPPLEMENTAL ASSAY METHOD

FOR

TITRATION OF BOVINE ROTAVIRUS ANTIBODY

(Constant Virus - Varying Serum Method)

A. SUMMARY

This is an *in vitro* assay method which employs a cell culture system for determining the antibody titer of serum against Group A bovine rotavirus.

B. MATERIALS

1. Cell Cultures

Multiple 96-well disposable plates are seeded (0.2 ml/well) with rhesus monkey kidney (MA-104) cells. Cells must be free of extraneous agents. The cells are seeded at a density that will produce 90-100% confluency after two days of incubation.

2. Growth Medium

The cells are grown in Minimum Essential Medium (MEM) with 7% fetal bovine serum and additives (Appendix, 1.) at a temperature of 35 to 37°C in an incubator containing an atmosphere of 5% carbon dioxide (CO_2) and a relative humidity of 70 to 80%. Growth medium is not changed unless excess acidity occurs or cells are not growing well.

3. Maintenance Medium

Maintenance medium (Appendix, 2.) without serum is used to rinse the cells prior to inoculation. It is also used as a diluent, in the presence of pancreatin*, for the serum-virus neutralization assay.

* Pancreatin 4XNF (10X), Gibco Laboratories, catalogue no. 610-5720AG. No endorsement expressed or implied.

4. Indicator Virus

The National Veterinary Services Laboratories (NVSL) reference bovine rotaviruses for serotypes 6 (NCDV-Lincoln strain) and 10 (B223 strain) are used as controls for the cell system.

5. Primary Antibody

When indirect immunofluorescence (IIF) and not cytopathic effect (CPE) is used to titer the virus, serotype- or strain-specific antisera or monoclonal antibodies are used as primary antibody.

6. Fluorescent Antibody Conjugate

NVSL reference fluorescein isothiocyanate-conjugated immunoglobulin-specific antiserum is used in the IIF assay.

C. METHOD

1. The indicator virus is diluted to contain 100 to 700 $TCID_{50}$ per 0.2 ml, using maintenance media containing a previously titrated amount of pancreatin, the maximum that the MA-104 cells will tolerate. This dilution is determined by previous titrations and is designated the "stock virus". The dilution factor is calculated by dividing the titer of the indicator virus by the desired titer of stock virus.

2. The serum to be assayed is heat-inactivated at 56°C for 30 minutes. Serial two-fold dilutions are made in sterile tubes with diluent containing pancreatin and mixed using a Vortex or similar type of mixer*.

3. An equal volume of stock virus and of each serum dilution are added together, mixed, and allowed to incubate at 37°C for 60 minutes. The mixing of equal volumes of serum and virus results in a further two-fold dilution of serum.

4. Cells that have been seeded in 96-well plates four to six days previously are inverted and the growth medium removed by gentle shaking and tamping on sterile gauze. The cells are rinsed with 0.2 ml of maintenance medium per well, the medium again decanted from the plate, and the cells refed with 0.2 ml of maintenance media which remains on the plate for one hour at 37°C.

5. The final rinse is removed from the cells, as above. Each well is inoculated with 0.2 ml per well of each stock virus-serum dilution mixture, at a minimum of four wells per dilution. A minimum of eight wells remain uninoculated with virus, to serve as negative cell controls; they receive 0.2 ml per well of only the pancreatin-containing diluent.

*No endorsement expressed or implied.

6. The stock virus is back-titrated by preparing serial ten-fold dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) in diluent containing pancreatin. These are then mixed with an equal volume of diluent containing pancreatin and at least four wells per dilution are inoculated with 0.2 ml of the mixture.

7. The plates are incubated at 37°C, in an atmosphere of 5.0% CO_2 and high humidity, for five days. After five days, the cells can be examined for CPE typical of bovine rotavirus and the 50% endpoints calculated. The $TCID_{50}$ of the stock virus is calculated by the method of Spearman and Karber; its titer must be between 50 and 350 $TCID_{50}$ per 0.2 ml for a test to be valid. The cells in the negative control wells must remain normal.

8. Certain strains of bovine rotavirus may not exhibit pronounced CPE, thus an IIF assay may be necessary to determine their titer.

a. After the medium is decanted, the cells are gently rinsed in phosphate buffered saline (PBS), then in deionized water. The cells are fixed in a solution of 80% acetone-20% deionized water at 4°C for 15 minutes. The acetone is discarded and the plates air-dried.

b. The wells are covered with 0.1 ml per well of a previously-titrated dilution of specific primary antibody and held in a high humidity, 37°C incubator for 30 minutes. Excess primary antibody is washed from the plates by two gentle PBS rinses and one deionized water rinse. The plates are shaken gently and lightly touched to an absorbent towel to remove excess moisture.

c. While still moist, wells are covered with 0.1 ml per well of species-specific, conjugated anti-immunoglobulin antiserum. Again, the plates are incubated at 37°C for 30 minutes. Washing is repeated, as in step b. The plates are air-dried, face-down.

d. The cell monolayers are examined by fluorescent microscopy using a Ploem illuminator and blue light (Xenon lamp). Any cells showing immunofluorescence characteristic of bovine rotavirus are considered positive and the 50% endpoints calculated. The $TCID_{50}$ of the stock virus is calculated using the Spearman-Karber method; its titer must be between 50 and 350 $TCID_{50}$ per 0.2 ml for a test to be valid. Also, the non-inoculated wells must be negative for immunofluorescence.

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Appendix

1. **Growth Medium**

MEM (Eagle) with Earle's salts*	1.0 packet
Deionized water q.s.	1.0 liter
Sodium bicarbonate	2.2 gram
Gentamicin sulfate	50.0 mg
Penicillin	25,000 U
Streptomycin	100.0 mg
Heat inactivated or irradiated fetal bovine serum	70.0 ml
200 mM L-Glutamine (100X)	292.0 mg
0.22 micron filtration	

2. **Maintenance Medium**

MEM (Eagle) with Earle's salts*	1.0 packet
Deionized water q.s.	1.0 liter
Sodium bicarbonate	2.2 gram
Gentamicin sulfate	50.0 mg
Amphotericin B	5.0 mg
Penicillin	100,000 U
Streptomycin	100.0 mg
200 mM L-Glutamine (100X)	292.0 mg
0.22 micron filtration	

* MEM with Earle's salts, Gibco Laboratories, catalogue no. 410-1500EB. No endorsement expressed or implied.